METHODS AND MEANS FOR DELAYING SEED SHATTERING IN PLANTS

Field of the invention

The invention relates to plant genetic engineering. In particular, the invention relates to methods and compositions for modulating properties of fruit dehiscence in plants, particularly *Brassicaceae* plants, specifically to improved methods and means for reducing seed shattering, or reducing seed shattering until after harvest, in plants such as *Brassicaceae* plants, particularly the *Brassicaceae* plants grown for oil production, to a degree which is agronomically important.

Background

Siliques or pods from *Brassicaceae* plants release their seeds through a process called dehiscence. A silique consists of two carpels joined margin to margin. The suture between the margins forms a thick rib, called replum. As pod maturity approaches, the two valves separate progressively from the replum, along designated lines of weakness in the pod, eventually resulting in the shattering of the seeds that were attached to the replum. The dehiscence zone defines the exact location of the valve dissociation.

Shedding of seed (also referred to as "seed shatter" or "pod shatter") by mature pods, before or during crop harvest, is a universal phenomenon with crops that develop dry dehiscent fruits. Premature seed shatter results in a reduced seed recovery, which represents a problem in crops which are grown primarily for the seeds, such as oil producing *Brassicaceae* plants, particularly oilseed rape. Another problem related to premature seed shattering is an increase in volunteer growth in the subsequent crop year. In oilseed rape, pod-shatter related yield losses typically range from 10 to 25%, but can reach up to 50%, depending on the weather conditions.

Current commercial oilseed rape varieties are extremely susceptible to shattering. Kadkol *et al.* [(1986), Aust. J. Biol. 34: 79] reported increased resistance towards

shattering in a single, Australian accession of rape. Variation in pod maturation has further been observed in mutants of rape stemming from irradiated seeds [Luczkiewicz (1987), Proc. 7th Int. Rapeseed Congress 2: 463]. However the traditional methods for breeding have been unsuccessful in introducing shatter resistance into rape cultivars, without interference in other desirable traits such as early flowering, maturity and blackleg resistance [Prakash and Chopra (1990), Genetical Research 56: 1].

Several genes, which promote or inhibit pod dehiscence have been identified in *Arabidopsis thaliana* through mutant analysis. These genes are encoding putative MADS box and basic helix-Loop-Helix mutants. Combined mutants in both *SHATTERPROOF1* (*SHP1*; initially referred to as *AGL1*) and *SHATTERPROOF2* (*SHP2*; initially referred to as *AGL5*) result in indehiscent siliques (i.e. siliques which remain closed upon maturity in *Arabidopsis thaliana*) (Liljegren *et al.*, 2000, Nature 404, 766-770). Similarly, mutants in the *INDEHISCENT* gene in *Arabidopsis thaliana* (PCT publication WO 01/79517), as well as in *ALCATRAZ* (Rajani *et al.* 2001, Current Biology 11, 1914-1922) interfered with pod dehiscence leading to podshatter resistance. Constitutive expression of *FRUITFUL* (*FUL*), a repressor of *SHP* and *IND*, in *Arabidopsis thaliana* also resulted in indehiscent siliques (Ferrandiz *et al.*, 2000, Science, 289, 436-438). These transcription factors are thus regulating the development of the valves, including the valve margins and the dehiscence zone.

Genes for a number of hydrolytic enzymes, such as endopolygalacturonases, which play a role, during pod dehiscence, in the programmed breakdown of the dehiscence zone in pods from *Brassicaceae* plants have also been identified (see e.g. WO 97/13865; Petersen *et al.*, Plant. Mol. Biol., 1996, 31:517-527).

To isolate mutant alleles corresponding to *ind*, *alc* or *shp1-shp2* in economically important *Brassicaceae* plants, such as oilseed rape, is a laborious and time consuming task. Moreover, such isolation may be complicated by the amphidiploidy in oilseed rape and the consequent functional redundancy of the corresponding genes.

It has been described that the expression of the *ALC, IND, AGL1* and *AGL5* genes or their homologues, may be downregulated using gene silencing techniques such as antisense suppression or cosuppression (WO99/00503; WO01/79517; WO0159122).

Vancanneyt *et al.*, 2002 (XIII International Conference on *Arabidopsis* Research, Sevilla, Spain June 28-July 2; 2002) reported that the expression of *FUL* from *A. thaliana* under control of a CaMV 35S promoter in oilseed rape, resulted in a number of podshatter resistant transformants. Pods of such podshatter resistant lines had no dehiscence zone, and opening of the pods could only be achieved by random fracture of the valves by applying considerable pressure.

Vancanneyt *et al.*, 2002 (XIII International Conference on *Arabidopsis* Research, Sevilla, Spain June 28-July 2; 2002) also reported that silencing of the *IND* gene in *Arabidopsis thaliana* using so-called dsRNA silencing techniques resulted in almost complete podshatter resistance. 98% of the transgenic *Arabidopsis* lines developed siliques, which did not open along the valve suture, and could only be opened by applying considerable pressure to the valves.

Furthermore, experiments described in this application (see below) indicate that silencing of the IND gene in *Brassica napus* using dsRNA gene silencing techniques, whereby the dsRNA sequence is identical to one of the *IND* homologues of *Brassica napus*, results in podshatter resistant lines with pods that bearly open by random fracture of the valves in a standardized random impact test.

It is important to realize that while seed shattering constitutes an important problem in oilseed rape culture, which may be solved by developing podshatter resistant lines, ultimately, separation of the seeds from the pods is still required. In normal agricultural practice this is achieved by threshing of the pods by a combine harvester. Threshing of the pods by a combine harvester must be complete and must cause minimum damage to the seeds thus released. However, as pod strength increases, the more severe action required to thresh them causes an unacceptable level of damage to the seed. The pods of podshatter resistant *Brassicaceae* plants should thus not be so strong that they cannot be threshed in a combine harvester (Bruce *et al.* 2001, J. Agric. Engng Res. 80, 343-350).

The art thus remains defective in providing method and means for reducing seed shattering in *Brassicaceae* plants, particularly in oilseed rape, while retaining a sufficient threshability.

These and other objects are achieved by the present invention, as indicated by the various embodiments described in the summary of the invention, detailed description and claims.

Summary of the invention

In one embodiment of the invention, a method is provided for reducing seed shattering in a plant, preferably a *Brassicaceae* plant, such as an oilseed rape plant, comprising the steps of:

- creating a population of transgenic lines of the plant, preferably the *Brassicaceae* plant, wherein the transgenic lines of the population exhibit variation in podshatter resistance, and wherein the population is obtainable by
    - introducing a chimeric gene into cells of the plant, preferably the *Brassicaceae* plant, to create transgenic cells, the chimeric gene comprising the following operably linked DNA fragments:
        - a plant-expressible promoter;
        - a DNA region which when transcribed yields a double-stranded RNA molecule capable of reducing the expression of a gene endogenous to the plant, preferably the *Brassicaceae* plant, the gene being involved in the development of a dehiscence zone and valve margin of a pod of the plant, preferably the *Brassicaceae* plant, such as a gene selected from the group of *INDEHISCENT* gene from *Arabidopsis thaliana*, *ALCATRAZ* gene from *Arabidopsis thaliana*, *SHATTERPROOF*1 gene from *Arabidopsis thaliana*, *SHATTERPROOF*2 gene from *Arabidopsis thaliana* or a homologous gene thereof present in the *Brassicaceae* plant , and the RNA molecule comprising a first and second RNA region wherein
            (i) the first RNA region comprises a nucleotide sequence of at least 19 consecutive nucleotides having about 94% sequence identity to the nucleotide sequence of the endogenous gene

involved in the development of a dehiscence zone and valve margin of the pod;

(ii) the second RNA region comprises a nucleotide sequence complementary to the 19 consecutive nucleotides of the first RNA region;

(iii)the first and second RNA region are capable of base-pairing to form a double stranded RNA molecule between at least the 19 consecutive nucleotides of the first and second region;

(b) a 3' end region comprising transcription termination and polyadenylation signals functioning in cells of the plant;

wherein the chimeric gene, when expressed in cells of the plant, preferably the *Brassicaceae* plant increases podshatter resistance compared to podshatter resistance in an untransformed plant, preferably an untransformed *Brassicaceae* plant, while maintaining an agronomically relevant threshability of the pods of the plant;

- regenerating transgenic lines from the transgenic cells; and
- selecting a podshatter resistant plant from the population wherein the plant has pods exhibiting delayed seed shattering.

Agronomically relevant threshability preferably corresponds to a half-life time of the pods in a Random Impact test between 10 and 60 seconds, preferably between 40 and 60 seconds.

In an embodiment of the invention, the plant expressible promoter may be a relatively weak plant expressible promoter such as an opine synthetase promoter from *Agrobacterium spp.*, a promoter selected from a nopaline synthetase promoter, an octopine synthetase promoter, a agrocinopine synthetase promoter or a mannopine synthetase promoter, or a dehiscence zone or valve margin selective promoter and the sense and antisense RNA region may comprises a nucleotide sequence of about 19 to about 500 consecutive nucleotides having a sequence similarity of about 90% to about 100% to the nucleotide sequence of the endogenous gene.

In another embodiment of the invention the first RNA region may comprise a sequence of about 50 to about 500 consecutive nucleotides having about 50% to about 88%, preferably about 65% to about 75% sequence identity with the endogenous gene; the second RNA may comprise a nucleotide sequence having about 90 to about

100% sequence similarity to the complement of the nucleotide sequence of the first RNA region; and the first and second RNA region are capable of forming a double stranded RNA region.

In yet another embodiment of the invention, the *Brassicaceae* plant may be an oilseed rape plant; the first RNA region may comprise a nucleotide sequence comprising at least 19 consecutive nucleotides from the nucleotide sequence of a second gene involved in the development of a dehiscence zone and valve margin of a pod, the second gene being endogenous to a *Brassicaceae* plant different from oilseed rape such as is a gene selected from the group of *INDEHISCENT* gene from *Arabidopsis thaliana*, *ALCATRAZ* gene from *Arabidopsis thaliana*, *SHATTERPROOF*1 gene from *Arabidopsis thaliana*, *SHATTERPROOF*2 gene from *Arabidopsis thaliana*; the second RNA comprises a nucleotide sequence having about 90 to about 100% sequence similarity to the complement of the nucleotide sequence of the first RNA region; and the first and second RNA region are capable of forming a double stranded RNA region.

In another embodiment of the invention, a method is provided for reducing seed shattering in an oilseed rape plant comprising the following steps:

(2) creating a population of transgenic lines of the oilseed rape plant, wherein the transgenic lines of the population exhibit variation in podshatter resistance, and wherein the population is obtainable by

(i) introducing a chimeric gene into cells of the oilseed rape plant, to create transgenic cells, the chimeric gene comprising the following operably linked DNA:

(a) a plant-expressible promoter;

(b) a DNA region which when transcribed yields a double-stranded RNA molecule capable of reducing the expression of a gene endogenous to the oilseed rape plant, the gene being involved in the development of a dehiscence zone and valve margin of a pod of the oilseed rape plant, such as a gene selected from the group of *INDEHISCENT* gene from *Arabidopsis thaliana*, *ALCATRAZ* gene from *Arabidopsis thaliana*, *SHATTERPROOF*1 gene from *Arabidopsis thaliana*, *SHATTERPROOF*2 gene from *Arabidopsis*

thaliana, and the RNA molecule comprising a first and second RNA region wherein

(i) the first RNA region comprises a nucleotide sequence of at least 50 consecutive nucleotides having at least about 90% sequence identity to the nucleotide sequence of a gene from *Arabidopsis thaliana* involved in the development of a dehiscence zone and valve margin of the pod;

(ii) the second RNA region comprises a nucleotide sequence complementary to the 50 consecutive nucleotides of the first RNA region;

(iii)the first and second RNA region are capable of base-pairing to form a double stranded RNA molecule between at least the 50 consecutive nucleotides of the first and second region;

(c) a 3' end region comprising transcription termination and polyadenylation signals functioning in cells of the plant;

(ii) regenerating transgenic lines from the transgenic cells; and

(3) selecting a podshatter resistant plant from the population wherein the plant has pods exhibiting reduced seed shattering.

The invention also relates to the chimeric genes according to the invention, as well as cells of a *Brassicaceae* plant and their seeds and progeny comprising such chimeric genes, and *Brassicaceae* plants obtainable by the methods according to the invention.

It is yet another object of the invention to provide isolated DNA fragments comprising a nucleotide sequence from SEQ ID No 2 or SEQ ID No 3 and isolated DNA fragments obtainable from a *Brassicaceae* plant, which hybridizes under stringent conditions to a DNA fragment comprising the nucleotide sequence of SEQ ID No 2 or No 3. The invention also relates to the use of such an isolated DNA fragment to reduce seed shatter or increase pod shatter resistance in *Brassicaceae* plants such as oilseed rape.

The invention also provides an agricultural method comprising the steps of

(i) sowing seeds according to the invention or planting plants according to the invention in a field;

(ii) growing the plants until the pods are mature;

(iii) harvesting seeds from the pods by threshing in a combine harvester.

Brief description of the Figures

Figure 1: alignment of the 5' end of the open reading frames from AT-*IND*; BN1-*IND* and BN2-*IND*

Detailed description of different embodiments of the invention

The invention is based on the unexpected observation that moderate dsRNA gene silencing of genes involved in the development of the dehiscence zone and valve margins of pods in *Brassicaceae* plants, particularly oilseed rape plants, allows the isolation of transgenic lines with increased pod shatter resistance and reduced seed shattering, the pods of which however may still be opened along the dehiscence zone by applying limited physical forces. This contrasts with transgenic *Brassicaceae* plants, wherein the dsRNA silencing is more pronounced, such as the ones described in the art, which result in transgenic lines with indehiscent pods, which no longer can be opened along the dehiscence zone, and which only open after applying significant physical forces by random breakage of the pods, whereby the seeds remain predominantly within the remains of the pods.

Moderate dsRNA gene silencing of genes can be conveniently achieved by e.g. operably linking the dsRNA coding DNA region to a relatively weak promoter region, or by e.g. choosing the sequence identity between the complementary sense and antisense part of the dsRNA encoding DNA region to be lower than 90% and preferably within a range of about 60 % to 80%.

Without intending to limit the invention to a particular mode of action, it is thought that the silencing of the expression level of the endogenous genes involved in dehiscence zone and valve margin development in these instances is incomplete, such that the expression of the endogenous gene in the presence of the gene-silencing chimeric genes according to the invention is about 5% to about 20%, particularly about 10% of the expression of the endogenous gene in the absence of the gene-silencing chimeric genes according to the invention.

Thus, in one embodiment of the invention, a method is provided for reducing seed shattering in a *Brassicaceae* plant by creation of a population of transgenic lines of a *Brassicaceae* plant, wherein said transgenic lines of said population exhibit variation in podshatter resistance. This population may be obtained by introducing a chimeric gene into cells of a *Brassicaceae* plant, to create transgenic cells, whereby the chimeric gene comprises a plant -expressible promoter and a 3' end region comprising transcription termination and polyadenylation signals functioning in cells of a *Brassicaceae* plant, operably linked to a DNA region which when transcribed yields a double-stranded RNA molecule capable of reducing the expression of a gene endogenous to a *Brassicaceae* plant, involved in the development of a dehiscence zone and valve margin of a pod of said *Brassicaceae* plant. The RNA molecule comprises a first (sense) RNA region and second (antisense) RNA region whereby

- the first RNA region comprises a nucleotide sequence of at least 19 consecutive nucleotides having about 94% sequence identity to the nucleotide sequence of the endogenous gene;
- the second RNA region comprises a nucleotide sequence complementary to the at least 19 consecutive nucleotides of the first RNA region;
- the first and second RNA region are capable of base-pairing to form a double stranded RNA molecule between the mentioned at least 19 consecutive nucleotides of the first and second region.

Expression of the chimeric gene in *Brassicaceae* plants increases podshatter resistance compared to podshatter resistance in an untransformed *Brassicaceae* plant, while however maintaining an agronomically relevant threshability of the pods. After regeneration of transgenic lines from the transgenic cells comprising the chimeric genes according to the invention, a podshatter resistant plant can be selected from the generated population.

As used herein, «a gene involved in development of a dehiscence zone and valve margins of a pod» refers to a gene, which when mutated in a plant, leads to the development of pods wherein the dehiscence zone and/or the suture of the pod is less or not developed, either alone, or in combination with mutations in other such genes. These genes include the *INDEHISCENT* gene from *Arabidopsis thaliana*, the *ALCATRAZ* gene from *Arabidopsis thaliana*, the *SHATTERPROOF*1 gene from *Arabidopsis thaliana* or the *SHATTERPROOF*2 gene from *Arabidopsis thaliana* or a

homologous gene thereof. These genes encode proteins comprising MADS box or basic Helix-Loop-Helix domains and are believed to be transcriptional activators.

Nucleotide sequences of these genes or parts thereof can be found in databases and have the following accession numbers: AT-*SHP*1 (M55553; AV557878; AV556852); AT-*SHP*2 (M55550; BG459390): AT-*IND* (AX320925); a homologue of AT-*IND* from *Brassica rapa subsp. pekinenis* (AT002234); AT-*ALC* (AX211753; AX211755; AX211760). All these nucleotide sequences are hereby incorporated specifically by reference.

DNA fragments comprising variant sequences of the above mentioned nucleotide sequences can be isolated from other *Brassicaceae* plants, particularly from other oilseed rape plants, subspecies or varieties by methods commonly known in the other. These methods include hybridization under stringent conditions of genomic or cDNA libraries derived from such other plants, subspecies or varieties with a probe consisting of part or all of any of the above mentioned genes.

Such variants of genes involved in dehiscence zone and valve margin development or suitable parts thereof may also be identified and isolated using e.g. polymerase chain reaction amplification using an appropriate pair of oligonucleotides having at least about 16 contiguous nucleotides, particularly at least about 50 contiguous nucleotides, more particularly at least about 100 contiguous nucleotides of the nucleotide sequence of the above mentioned genes involved in dehiscence zone and valve margin development. Preferably, the sequence of the oligonucleotides is derived from parts of the nucleotide sequence of the genes involved in development of dehiscence zone and valve margins of pods of *Brassicaceae* plants, which are different from those parts of these genes encoding common protein motives such as MADS boxes or K-regions or basic helix-loop-helix regions. Examples of PCR amplified sequences of genes involved in the development of dehiscence zone and valve margins of pods of *Brassicaceae* plants are DNA molecules having the sequence of SEQ ID NO 2 or SEQ ID No 3. Examples of nucleotide sequences of suitable oligonucleotides primers to amplify parts of genes involved in the development of dehiscence zone and valve margins of pods of *Brassicaceae* plants are represented in SEQ ID No 4 to SEQ ID No 8.

"Stringent hybridization conditions" as used herein mean that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C. Other hybridization and wash conditions are well known and are exemplified in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

As used herein, "a gene endogenous to a *Brassicaceae* plant" is a gene that naturally occurs in the species of the *Brassicaceae* family that has been chosen for modulation of the pod shatter resistance.

As used herein, « an agronomically relevant threshability » refers to the resistance of a pod, particularly an oilseed rape pod, to opening along the dehiscence zone of the pod with concurrent release of the seeds, upon application of physical forces that allow complete opening of the pods while preventing damage to the seeds, as they are used e.g. in a combine harvester. A good correlation has been found between the half-life time of pods in a random impact test and their threshability. Random impact tests (RITs) and algorithms to define the half-life time of pods in such RITs have been described in Bruce *et al.*, 2001 (J. Agric. Engng Res. 80, 343-350) and Morgan *et al.*, 1988 (Fields Crop Research 58, 153-165). Both publications are hereby incorporated by reference. Briefly, a sample of immature pods is placed in a closed drum together with steel balls, and the drum is then vigorously agitated for increasing periods of times (e.g. 5s, 10s, 20s, 40s). After each period, the drum is opened and the number of broken and damaged pods is counted. The most accurate estimation of the level of shattering resistance for each line is calculated by fitting a linear x linear curve to all the available data and estimating the time take for half of the pods within a sample to be broken ("pod shatter half life"). It is important however that pods open mainly along the dehiscence zone, and are not simply pulverized, as may occur with indehiscent pods. Pod shatter half lives of oilseed rape, as determined in a RIT, which

correspond to agronomically relevant threshability should not exceed 60 seconds. Typical values for control lines of commercially available oilseed rape varieties are about 10 seconds. Thus, lines with improved pod shatter resistance with agronomically relevant threshability have a pod shatter half life in RIT between about 10 and about 60 seconds, preferably between about 20 and about 60 seconds, particularly between about 40 and about 60 seconds.

In a particular embodiment of the methods of the invention, the moderate gene silencing can be achieved by employing a relatively weak plant expressible promoter.

As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly and indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g. enhancers), at which gene expression regulatory proteins may bind.

The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (i.e. regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a dNA sequence located upstream (I.e. 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (i.e. 3') of the coding sequence and comprises suitable transcription termination and or regulation signals, including one or more polyadenylation signals.

As used herein, the term "plant-expressible promoter" means a DNA sequence that is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e. certain promoters of viral or bacterial origin such as the CaMV35S promoter (Hapster *et al.* 1988, Mol. Gen. Genet. 212, 182-190), the subterranean clover virus promoter No 4 or No 7 (WO9606932) or T-DNA gene promoters.

As used herein "a relatively weak plant expressible promoter" is a promoter which initiates and controls transcription of the operably linked DNA fragments about 10 to about 100 times less efficient that an optimal CaMV35S promoter. Relatively weak plant expressible promoters include the promoters or promoter regions from the opine synthase genes of *Agrobacterium spp.* such as the promoter or promoter region of the nopaline synthase, the promoter or promoter region of the octopine synthase, the promoter or promoter region of the mannopine synthase, the promoter or promoter region of the agropine synthase and any plant expressible promoter with comparably activity in transcription initation. Other relatively weak plant expressible promoters may be dehiscence zone selective promoters, or promoters expressed predominantly or selectively in dehiscence zone and/or valve margins of *Brassicaceae* pods, particularly oilseed rape pods, such as the promoters described in WO97/13865.

In this embodiment of the invention, the chimeric gene may comprise a transcribed DNA region encoding a dsRNA, wherein the sense and antisense RNA regions comprise a nucleotide sequence which has a high degree of sequence identity with the endogenous gene (or its complement) of the *Brassicaceae* plant, the pods of which are to rendered more resistant. Thus, sense and antisense RNA regions may comprise at least about 50, 100 , 200, 500 or more consecutive nucleotides having about 90 % to about 100% sequence identity to the endogenous gene of the *Brassicaceae* plant or its complement.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970) Computer-assisted sequence alignment, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA molecules will be clear from the context of the application.

In another embodiment of the invention, the moderate gene silencing can be achieved by employing a first and second RNA region that have a sequence identity to an endogenous gene involved in dehiscence zone and valve margin development of the pod, or part thereof, or their complementary sequences, ranging between about 60 to about 88% sequence identity, preferably ranging between about 60 to about 75 % sequence identity. The length of the sense and antisense RNA regions may be at least 50, 100, 200, 500 or more nucleotides.

It has been observed by alignment of the sense RNA region (or complement of the antisense region) of the *Arabidopsis thaliana* derived sequence of *IND* used in the chimeric genes of the Examples and the corresponding region of the nucleotide sequences of the *Brassica napus IND* genes that there is not a single subregion of 21 consecutive nucleotides which are either identical or have only one mismatch between each of the *Brassica napus* sequences and the *Arabidopsis thaliana* sequence (see Fig 2). The sequences share however several subsequences of 19 consecutive nucleotides with only one mismatch (i.e. having a sequence identity of about 94%). Although not intending to limit the invention to a particular mode of action, it is thought that the absence of nucleotide sequence "words" of 21 nucleotides shared between the dsRNA molecule and the endogenous gene may play an important role in the moderation of the gene silencing, as these 21-mers are generally expected to play an important role in the initiation and maintenance of dsRNA mediated post-transcriptional silencing.

For oilseed rape plants, a moderate gene-silencing response can be conveniently achieved by using a chimeric gene encoding a dsRNA molecule, wherein the sense and antisense region comprise a nucleotide sequence having about 90% or 95% sequence identity, particularly are identical to a nucleotide sequence of at least 19 consecutive nucleotides from a gene involved in dehiscence zone and valve margin development of the silique from *Arabidopsis thaliana*. The nucleotide sequence may

however be longer than 19 nucleotides and may be 50, 100, 200, 500 or even more nucleotides in length.

In a particular embodiment of the invention, reduced seed shattering in oilseed rape can be achieved by using a chimeric gene encoding a dsRNA molecule wherein the sense and antisense regions correspond to the nucleotide sequence of the *IND* gene of *Arabidopsis thaliana* (SEQ ID No 1) from nucleotide +12 to +223 relative to the ATG startcodon of the *Arabidopsis thaliana IND* gene or its complement, i.e. from the nucleotide at position 27 to the nucleotide at position 237 in SEQ ID No 1.

dsRNA encoding chimeric genes according to the invention may comprise a heterologous intron located in the spacer sequence between the sense and antisense RNA regions in accordance with the disclosure of WO 99/53050 (incorporated herein by reference).

The invention also provides novel isolated DNA fragments comprising the nucleotide sequence of SEQ ID No 2 OR SEQ ID No 3. and the use thereof for modulating or increasing podshatter resistance in plants, such as *Brassicaceae* plants, particularly oilseed rape plants.

Preferred *Brassicaceae* to be treated in accordance with this invention, besides *Brassica napus,* include *Brassica juncea, Brassica oleraceae, Brassica carinata, Brassica nigra, Brassica campestris* and the like, and any intergenic crosses or synthetic varieties thereof. Other *Brassicaceae*, which may be treated according to the methods of the inventions include *Brassica cretica* (mustard), *Brassica elongata* (elongated mustard), *Brassica narinosa* (broadbeaked mustard), *Brassica nigra* (black mustard), *Brassica rapa* (field mustard), *Brassica rupstris* (mustard), Brassica *tournefortii* (Asian mustard). *Brassica napus* (2n=38, genome AACC) is an amphidiploid species, which originated from a spontaneous hybridization of *Brassica rapa* L. (syn. *B. campestris*; 2n=20, AA) and *Brassica oleracea* L. (2n=18, CC). B. napus contains the complete chromosome sets of these two diploid genomes.

As used herein, « a plant from the family *Brassicaceae* » or « a *Brassicaceae* plant » is a plant which according to the current botanical standard would be classified into

the family *Brassicaceae* (formerly *Cruciferaeae*). *Brassicaceae* (Mustard) family members are easy to distinguish. They are annual or perannual plants with alternate leaves without stipules and posses simple inflorescence or branched racemes. The flowers are bilaterally symmetrical and hypogynous. With few exceptions, the flowers have 4 petals (free) alternating with 4 sepals (free) ; 6 stamens (4 long and 2 short), an ovary of 2 united carpels with parital placenta, 2 locular through the formation of a membranous false septum ; fruit is a dehiscent capsule opening by 2 valves. *Brassicaceae* include *inter alia* the following genera : *Sisymbrium, Descurania, Alliaria, Arabidopsis, Myagrum, Isatis, Bunia, Erysium, Hesperis, Malcolmia, Matthiola, Chorispora, Euclidium, Barbarea, Rorippa, Armoracia, Nasturtium, Dentaria, Cardamine, Cardaminopsis, Arabis, Lunaria, Alyssum, Berteroa, Lobularia, Draba, Erophila, Cochlearia, Camelina, Neslia, Capsella, Hornungia, Thlsapi, Iberis, Lepidium, Cardaria, Coronopus, Subularia, Conringia, Diplotaxis, Brassica, Sinapsis, Eruca, Erucastrum, Coincya, Hirschfeldia, Cakile, Rapistum, Crambe, Enarthrocarpus, Rhaphanus and Clausia*.

« Oilseed rape » as used herein, should be understood to include the species *Brassica napus, Brassica junceae* and *Brassica campestris*.

The means and methods of the invention may also be used in plants other than Brassicaceae, specifically plants which have fruits or pods wherein valves have to separate to release the seeds. These include members of the family *Fabaceae*, such as pea, beans, soybean and the like.

It is also an object of the invention to provide plant cells and plants containing the chimeric genes or the RNA molecules according to the invention. Gametes, seeds (including crushed seeds and seed cakes), embryos, either zygotic or somatic, progeny or hybrids of plants comprising the chimeric genes of the present invention, which are produced by traditional breeding methods are also included within the scope of the present invention.

The plants obtained by the methods described herein may be further crossed by traditional breeding techniques with other plants to obtain podshatter resistant progeny plants comprising the chimeric genes of the present invention.

The following non-limiting Examples describe the construction of chimeric genes for reducing seed shattering or modulating fruit dehiscence in *Brassicaceae* plants, and *Brassicaceae* plants comprising such chimeric genes, exhibiting reduced seed shattering in agronomically relevant ranges.

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No. 1: nucleotide sequence of the *INDEHISCENT* gene of *A. thaliana* (AT-*IND*)

SEQ ID No. 2 : nucleotide sequence of a *INDEHISCENT* homologue from *Brassica napus* (BN1-*IND*)

SEQ ID No. 3 : nucleotide sequence of a second *INDEHISCENT* homologue from *Brassica napus* (BN2-*IND*)

SEQ ID No. 4 : common nucleotide sequence of oligonucleotides CO109/CO111

SEQ ID No. 5 : common nucleotide sequence of oligonucleotides CO110/CO112

SEQ ID No. 6 : common nucleotide sequence of oligonucleotides CO113/CO114

SEQ ID No. 7 : common nucleotide sequence of oligonucleotides CO115/CO117

SEQ ID No. 8 : common nucleotide sequence of oligonucleotides CO116/CO118

SEQ ID No. 9: nucleotide sequence of the *SHATTERPROOF* 1 gene of A. *thaliana* (AT-*SHP*1)

SEQ ID No. 10: nucleotide sequence of the *SHATTERPROOF* 2 gene of A. *thaliana* (AT-*SHP*2)

SEQ ID No. 11: nucleotide sequence of the *ALCATRAZ* gene of A. *thaliana* (AT-*ALC*)

Examples

Example 1: Construction of chimeric genes encoding dsRNA capable of reducing the expression of a gene involved in dehiscence zone and valve margin development and introduction into plants.

A. Based on *IND* gene sequences

A 5' end fragment of the *IND* gene of *Arabidopsis thaliana* or of the homologous genes from *Brassica napus* was amplified by PCR under standard conditions, using as template genomic DNA from *Arabidopsis thaliana* or PCR amplified DNA from *B. napus* comprising the sequences of SEQ ID No 2 or SEQ ID No 3 and the following oligonucleotides:

- For the 5' end of the At-*IND* gene: an oligonucleotide comprising the sequence of SEQ ID No 7 and an oligonucleotide comprising the sequence of SEQ ID No 8, equipped with appropriate restriction enzyme sites at the 5' end of the oligonucleotides to allow directional cloning.
- For the 5' end of the BN1-*IND* gene: an oligonucleotide comprising the sequence of SEQ ID No 4 and an oligonucleotide comprising the sequence of SEQ ID No 6, equipped with appropriate restriction enzyme sites at the 5' end of the oligonucleotides to allow directional cloning.
- For the 5' end of the BN2-*IND* gene: an oligonucleotide comprising the sequence of SEQ ID No 4 and an oligonucleotide comprising the sequence of SEQ ID No 5, equipped with appropriate restriction enzyme sites at the 5' end of the oligonucleotides to allow directional cloning.

The amplified PCR fragments of the homologous genes from *B. napus* have about 90% sequence identity, whereas the sequence identity between the At-Ind gene and each of the BN-Ind genes is about 65% (see Fig 1).

Using standard cloning techniques the following chimeric genes encoding dsRNA capable of reducing the expression of a gene involved in dehiscence zone and valve margin development of a pod have been constructed and introduced into T-DNA

vectors, together with an appropriate selective marker gene (plant-expressible bar gene).

pTCO219

pTCO219 is a T-DNA vector comprising between the T-DNA borders

1. A dsRNA encoding chimeric gene (p35S-dsRNA/At*IND*) comprising the following operably linked DNA fragments:
   - **p35S**: Promoter region from the Cauliflower Mosaic Virus 35S (Odell *et al.*(1985) Nature 313 : 810-812)
   - **At*IND*^{sense}**: A 211 bp fragment from nucleotide +12 to +223 relative to the ATG startcodon of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 27 to the nucleotide at position 239). This fragment is cloned in sense orientation.
   - **Pdk-intron**: Sequence of the second intron from the pyruvate orthophosphate dikinase gene (termed pdk gene) from Flaveria trinervia (Rosche & Westhoff (1995) Plant Molecular Biology 29-4 : 663-678)
   - **At*IND*^{antisense}**: A 211 bp fragment from nucleotide +12 to +223 relative to the ATG startcodon of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 27 to the nucleotide at position 239). This fragment is cloned in antisense orientation.
   - **3'ocs**: the 3' untranslated end from the octopine synthase gene (De Greve *et al.* (1982) J. Mol. Appl. Genet. 1: 499-512 ; Gielen *et al.* (1984) EMBO J. 3: 835-846).

2. A chimeric bar gene comprising the following operably linked DNA fragments:
   - **pSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers *et al.*, 1988, Plant Mol. Biol. 11: 745-759).

- ***bar***: the coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987, EMBO 6, 2519).
- **3'g7**: A 211bp DNA fragment containing the 3'end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTiB6S3 (Dhaese *et al.*, 1983, EMBO 2: 419; Velten and Schell, 1985, Nucl. Acids Res. 13: 6981).

Plasmid pTCO219 is derived from pGSV1. The basic intermediate vector pGSV1 has essentially been derived from pGSC1700 (Cornelissen and Vandewiele, 1989) and comprises the following structural elements:

- the plasmid core comprising the origin of replication from the plasmid pBR322 (*Bolivar et al.*, 1977) for replication in *Escherichia coli* (pBRori) and a restriction fragment comprising the origin of replication from the *Pseudomonas* plasmid pVS1 (Itoh *et al.*, 1984) for replication in *Agrobacterium tumefaciens* (pVS1ori);
- a selectable marker gene conferring resistance to streptomycin and spectinomycin (Sm/Sp) for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens*;

An artificial T-region consisting of the left and right border sequences of the TL-DNA from pTiB6S3 and polylinker allowing the insertion of GOI between the T-DNA border.

<u>pTKC89</u>

pTKC89 a is a T-DNA vector, similar to pTCO219, wherein the CaMV35S promoter has been exchanged for a nopaline synthase promoter *from Agrobacterium tumefaciens*, through standard cloning techniques.

<u>pTCO212</u>

pTCO212 is a T-DNA vector comprising between the T-DNA borders

1. A dsRNA encoding chimeric gene (p35S-dsRNA/BN2-*IND*) comprising the following operably linked DNA fragments:

- **p35S**: Promoter region from the Cauliflower Mosaic Virus 35S (Odell *et al.*(1985) Nature 313 : 810-812).
- **BN2-*IND*^{sense}**: A 261 bp fragment from nucleotide +12 to +273 relative to the ATG startcodon of a *Brassica napus* homolog (homolog 2) of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 30 to the nucleotide at position 290). This fragment is cloned in sense orientation.
- **Pdk-intron**: Sequence of the second intron from the pyruvate orthophosphate dikinase gene (termed pdk gene) from Flaveria trinervia (Rosche & Westhoff (1995) Plant Molecular Biology 29-4 : 663-678)
- **BN2-*IND*^{antisense}**: A 261 bp fragment from nucleotide +12 to +273 relative to the ATG startcodon of a *Brassica napus* homolog (homolog 2) of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 30 to the nucleotide at position 290). This fragment is cloned in antisense orientation.
- **3'ocs**: the 3' untranslated end from the octopine synthase gene (De Greve *et al.* (1982) J. Mol. Appl. Genet. 1: 499-512 ; Gielen *et al.* (1984) EMBO J. 3: 835-846).

2. A chimeric bar gene comprising the following operably linked DNA fragments:
   - **pSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers *et al.*, 1988, Plant Mol. Biol. 11: 745-759).
   - ***bar***: the coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987, EMBO 6, 2519).
   - **3'g7**: A 211bp DNA fragment containing the 3'end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTiB6S3 (Dhaese *et al.*, 1983, EMBO 2: 419; Velten and Schell, 1985, Nucl. Acids Res. 13: 6981).

PTCO212 is derived from pGVS1 (see above)

pTCO218

pTCO218 is a T-DNA vector comprising between the T-DNA borders

1. A dsRNA encoding chimeric gene (p35S-dsRNA/BN1-*IND*) comprising the following operably linked DNA fragments:
   - **p35S**: Promoter region from the Cauliflower Mosaic Virus 35S (Odell *et al.*(1985) Nature 313 : 810-812).
   - **BN1-*IND*^{sense}**: A 269 bp fragment from nucleotide +12 to +281 relative to the ATG startcodon of a *Brassica napus* homolog (homolog 1) of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 31 to the nucleotide at position 299). This fragment is cloned in sense orientation.
   - **Pdk-intron**: Sequence of the second intron from the pyruvate orthophosphate dikinase gene (termed pdk gene) from Flaveria trinervia (Rosche & Westhoff (1995) Plant Molecular Biology 29-4 : 663-678)
   - **BN1-*IND*^{antisense}**: A 269 bp fragment from nucleotide +12 to +281 relative to the ATG startcodon of a *Brassica napus* homolog (homolog 1) of the *Arabidopsis thaliana* Ind1 gene , encoding a Basic Helix Loop Helix protein involved in pod dehiscence (corresponding to the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 31 to the nucleotide at position 299). This fragment is cloned in antisense orientation.
   - **3'ocs**: the 3' untranslated end from the octopine synthase gene (De Greve *et al.* (1982) J. Mol. Appl. Genet. 1: 499-512 ; Gielen *et al.* (1984) EMBO J. 3: 835-846).

2. A chimeric bar gene comprising the following operably linked DNA fragments:

- **pSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers *et al.*, 1988, Plant Mol. Biol. 11: 745-759).
- ***bar***: the coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987, EMBO 6, 2519).
- **3'g7**: A 211bp DNA fragment containing the 3'end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTiB6S3 (Dhaese *et al.*, 1983, EMBO 2: 419; Velten and Schell, 1985, Nucl. Acids Res. 13: 6981).

PTCO218 is derived from pGVS1 (see above)

B. Based on *SHP1 (SHP2)* gene sequences.

Two sequence regions, indicated AB and CD respectively from the *SHP1* gene of A. *thaliana*, have been amplified by PCR and have been used to construct TDNA vectors containing chimeric dsRNA encoding genes capable of reducing the expression of *SHP* genes. The region indicated as "AB" has 100% sequence identity with *SHP1* and about 88% sequence identity with *SHP2*, whereas the region indicated as "CD" has 100% sequence identity with *SHP1* and about 77% sequence identity with *SHP2*.

pTCO233

pTCO233 is a T-DNA vector comprising between the T-DNA borders

1. A dsRNA encoding chimeric gene (p35S-dsRNA/At-*SHP*1^{AB}) comprising the following operably linked DNA fragments:
    - **p35S**: Promoter region from the Cauliflower Mosaic Virus 35S (Odell *et al.*(1985) Nature 313 : 810-812).
    - **At-*SHP*1/AB^{sense}**: A fragment of the *Arabidopsis thaliana SHP*1 gene corresponding to the nucleotide sequence of SEQ ID No 9 from the nucleotide at position 258 to the nucleotide at position 375. This fragment is cloned in sense orientation.

- **Pdk-intron**: Sequence of the second intron from the pyruvate orthophosphate dikinase gene (termed pdk gene) from Flaveria trinervia (Rosche & Westhoff (1995) Plant Molecular Biology 29-4 : 663-678)
- **At-*SHP*1/AB^{antisense}**: A fragment of the *Arabidopsis thaliana SHP*1 gene corresponding to the nucleotide sequence of SEQ ID No 9 from the nucleotide at position 258 to the nucleotide at position 375. This fragment is cloned in antisense orientation.
- **3'ocs**: the 3' untranslated end from the octopine synthase gene (De Greve *et al.* (1982) J. Mol. Appl. Genet. 1: 499-512 ; Gielen *et al.* (1984) EMBO J. 3: 835-846).

2. A chimeric bar gene comprising the following operably linked DNA fragments:
   - **pSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers *et al.*, 1988, Plant Mol. Biol. 11: 745-759).
   - ***bar***: the coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987, EMBO 6, 2519).
   - **3'g7**: A 211bp DNA fragment containing the 3'end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTiB6S3 (Dhaese *et al.*, 1983, EMBO 2: 419; Velten and Schell, 1985, Nucl. Acids Res. 13: 6981).

PTCO233 is derived from pGVS1 (see above).

pTCO234

pTCO234 is a T-DNA vector comprising between the T-DNA borders

1. A dsRNA encoding chimeric gene (p35S-dsRNA/At-*SHP*1^{CD}) comprising the following operably linked DNA fragments:
   - **p35S**: Promoter region from the Cauliflower Mosaic Virus 35S (Odell *et al.*(1985) Nature 313 : 810-812).

- **At-*SHP*1/CD^{sense}**: A fragment of the *Arabidopsis thaliana SHP*1 gene corresponding to the nucleotide sequence of SEQ ID No 9 from the nucleotide at position 567 to the nucleotide at position 726). This fragment is cloned in sense orientation.
- **Pdk-intron**: Sequence of the second intron from the pyruvate orthophosphate dikinase gene (termed pdk gene) from Flaveria trinervia (Rosche & Westhoff (1995) Plant Molecular Biology 29-4 : 663-678)
- **At-*SHP*1/CD^{antisense}**: A fragment of the *Arabidopsis thaliana SHP*1 gene corresponding to the nucleotide sequence of SEQ ID No 9 from the nucleotide at position 567 to the nucleotide at position 726). This fragment is cloned in antisense orientation.
- **3'ocs**: the 3' untranslated end from the octopine synthase gene (De Greve *et al.* (1982) J. Mol. Appl. Genet. 1: 499-512 ; Gielen *et al.* (1984) EMBO J. 3: 835-846).

2. A chimeric bar gene comprising the following operably linked DNA fragments:
   - **pSSuAra**: a 1726bp DNA fragment of promoter and leader sequence from the *Arabidopsis thaliana* rbcS *ATS1A* gene (Krebbers *et al.*, 1988, Plant Mol. Biol. 11: 745-759).
   - ***bar***: the coding sequence of the bialaphos resistance gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987, EMBO 6, 2519).
   - **3'g7**: A 211bp DNA fragment containing the 3'end formation signals obtained from the 3' untranslated region of the TL-DNA gene 7 on pTiB6S3 (Dhaese *et al.*, 1983, EMBO 2: 419; Velten and Schell, 1985, Nucl. Acids Res. 13: 6981).

PTCO234 is derived from pGVS1 (see above).

The above T-DNA vectors were introduced into *Agrobacterium* pGV4000 . This host strain is a rifampicin resistant derivative of C58, cured for pTiC58 (C58C1RifR) (Van Larebeke *et al.*, 1974). The non-oncogenic acceptor Ti plasmid, pGV4000, is derived from the nopaline Ti plasmid pTiC58.The entire T-DNA region from pTiC58 has been substituted yielding pMP90 (Koncz and Schell ,1986). Plasmid pGV4000

has been derived from pMP90 by introducing a chloramphenicol resistance gene as described by Deblaere *et al.*, 1985.

The resulting *Agrobacterium* strains were used to transform *Arabidopsis* plants according to the floral dipping method (Bechtold et al 1993., C.R. Acad. Sci. Paris, Sciences de la vie/Life Sciences, 316, 1194-1199) or *Brassica napus* plants according to the hypocotyl explant inoculation method (essentially as described in De Block *et al*, 1989, Plant Physiol., 91: 64 or in WO 00/04173). All *Brassica napus* transformations were performed using a doubled haploid line derived from N90-740.

Example 2: Analysis of transgenic *Arabidopsis thaliana* lines and *Brassica napus* lines comprising the chimeric genes of Example 1.

A. Transgenic *Arabidopsis thaliana* lines.

Transgenic *Arabidopsis thaliana* lines were obtained by transformation using all of the T-DNA vectors of Example 1. The obtained lines were analyzed for podshatter resistance. Pods from untransformed wt *Arabidopsis* lines open spontaneously at maturity. In contrast, each of the population of transgenic lines, transformed using particular T-DNA vectors, contained transgenic lines, the plants of which developed pods which did not open spontaneously at maturity (indicated herein as podshatter resistant). The fraction of transgenic lines with podshatter resistance in the total population of lines transformed using a particular T-DNA vector varied between 23% and 98% (see Table 1). The physical forces needed to open closed mature pods of the podshatter resistant transgenic lines were determined in a semi-quantitive way by applying pressure between two fingers. Pods which completely open along the dehiscence zone at the slightest pressure were classified as "+", whereas pods which open only at the base of the dehiscence zone, and need harder pressure to open completely were classified as "++". Pods which can only be crushed (and do not open along the dehiscence zone) were classified as "+++".

From the results summarized in Table 1, the following conclusions can be drawn.

Using a dsRNA chimeric gene with a strong promoter and a sense/antisense region which is completely homologous to the endogenous *IND* gene of A. *thaliana* (pTCO219)

a) virtually all transgenic lines develop pods which remain closed at maturity
b) virtually all pods of these transgenic lines require considerable force to be opened.

Using a dsRNA chimeric gene with a relatively weak promoter and a sense/antisense region which is completely homologous to the endogenous *IND* gene of A. *thaliana* (pTKC89)

a) about 58% of transgenic lines develop pods which remain closed at maturity
b) about half of the pods of these transgenic lines require only slight to moderate physical forces to be opened.

Using a dsRNA chimeric gene with a strong promoter and a sense/antisense region which has about 65% sequence identity to the endogenous *IND* gene of A. *thaliana* (pTCO212 and pTCO218)

a) only about one third of the transgenic lines develop pods which remain closed at maturity
b) most of the pods of these transgenic lines require only slight to moderate physical forces to be opened.

Using a dsRNA chimeric gene with a strong promoter and a sense/antisense region which has about 100 % sequence identity to the endogenous *SHP*1 gene of A. *thaliana* and about 71% or 88 % sequence identity to the endogenous *SHP*2 gene of A. *thaliana* (pTCO233 and pTCO234)

a) only about one fourth to half of the transgenic lines develop pods which remain closed at maturity
b) most of the pods of these transgenic lines require only slight (pTCO233) to moderate physical forces (pTCO234) to be opened.

From these results it can be concluded that moderate gene silencing using either a relatively weak promoter, or a sense/antisense region that exhibits lower than 88%

and particularly around 65% sequence identity to a gene involved in dehiscence zone and valve margin development can be used to generate podshatter resistant *Arabidopsis thaliana* lines, all of the pods of which open along the dehiscence zone by application of only moderate physical forces.

Table 1. Podshatter resistance in transgenic *Arabidopsis* lines

DsRNA chimeric gene	T-DNA vector	Sequence similarity to target	# transformants analyzed	Podshatter resistant	Podshatter resistance after aplying pressure		
					+++	++	+
35S/AT-*IND*	PTCO219	100%	203	199 (98%)	199	0	0
NOS/AT-*IND*	PTKC89	100%	146	85 (58%)	36	19	30
35S/BN1-*IND*	PTCO218	65%	152	50 (33%)	20	12	18
35S/BN2-*IND*	PTCO212	65%	202	73 (36%)	20	20	33
35S/AT-*SHP*[AB]	PTCO233	100-88%	153	80 (52%)	19	43	17
35S/AT-*SHP*[CD]	PTCO234	100-71%	115	27 (23%)	7	7	13

B. Transgenic *Brassica napus* lines.

Transgenic *Brassica napus* lines were obtained by transformation using T-DNA vectors pTCO212, pTC0218 and pTCO219 of Example 1. Lines with single copy T-DNA insertions were selected. These lines were analyzed for podshatter resistance. Pods from untransformed wt *B. napus* lines remain normally closed at maturity, but open upon application of relatively mildly forces. To determine podshatter resistance, a Random Impact Test (described above) was used to determine the half life of the pods (results summarized in Table 2 and Figure 3).

Non-transgenic control lines have a pod half-life of about 10s in the RIT.

Using a dsRNA chimeric gene with a strong promoter and a sense/antisense region which has about 90 to about 100 % sequence identity to the endogenous *B. napus IND*

genes homologous to the *IND* gene of A. *thaliana* (pTCO212 and pTCO218), the pods never opened along the dehiscence zone and were pulverized after a long time in the drum. No meaningful half-life could be determined.

Using a dsRNA chimeric gene with a strong promoter and a sense/antisense region which has about 65 % sequence identity to the endogenous *B. napus IND* genes homologous to the *IND* gene of A. *thaliana* (pTCO219) pod half lives of about 15 to about 40 seconds in RIT were determined.

Table 2. Podshatter resistance in trangenic B. napus lines comprising the 35S-AT-*IND* chimeric gene (determined by RIT).

Pod line	construct	Half life s	lld50	uld50	Corrected lower 95%	Corrected upper 95%
Control	pTCO199	8.62	5.17	10.98	3.45	2.36
Control	WT	11.44	9.02	13.54	2.42	2.1
Control	pTCO199	11.93	8.45	15.52	3.48	3.59
Line 1	pTCO219	15.97	12.44	22.68	3.53	6.71
Line 2	pTCO219	16.43	12.27	25.57	4.16	9.14
Line 3	pTCO219	16.54	14.43	19.05	2.11	2.51
Line 4	pTCO219	18.55	15.13	22.51	3.42	3.96
Line 5	pTCO219	20.5	15.13	28.58	5.37	8.08
Line 6	pTCO219	31.29	26.21	39.65	5.08	8.36
Line 7	pTCO219	32	25.63	44.98	6.37	12.98
Line 8	pTCO219	42.7	33.61	57.45	9.09	14.75

From these results it can be concluded that the methods according to the invention, as exemplified in *Arabidopsis thaliana*, yield similar results in oilseed rape crop plants.